

Response of shallow subterranean freshwater amphipods to habitat drying

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Academic editor: Oana Moldovan | Received 19 October 2018 | Accepted 30 October 2018 | Published 16 November 2018

<http://zoobank.org/359479FF-F292-49B8-8B39-754E7E01739D>

Citation: Gilbert H, Keany J, Culver DC (2018) Response of shallow subterranean freshwater amphipods to habitat drying. Subterranean Biology 28: 15–28. <https://doi.org/10.3897/subtbiol.28.30700>

Abstract

The ability of three amphipods that occupy shallow subterranean habitats in the lower Potomac Basin of the US (hypotelminorheic), which often dry out seasonally, to withstand desiccation by burrowing in clay was investigated. Both *Crangonyx shoemakeri*, a wetland species, and *Stygobromus tenuis*, a subterranean species, burrowed in clay in the laboratory after surface water was removed. *Gammarus minus*, a spring species, did not. All three species exhibited behavioral changes as the habitat dried out.

Keywords

Adaptation to desiccation, burrowing, *Crangonyx shoemakeri*, *Gammarus minus*, Hypotelminorheic, shallow subterranean habitats, *Stygobromus*

Introduction

Freshwater habitats, such as wetlands and seasonal streams, typically first order streams, are subject to drying. Water level patterns, or hydroperiods, are an important way to categorize wetland habitats (Jackson et al. 2014, Batzer and Boix 2016). Permanent wetlands differ from seasonal wetlands (e.g., Scheffer 2004, Zimmer et al. 2016) in that their inhabitants are adapted to deal with a significant loss of water for months at a time (Wiggins et al. 1980). These adaptations include resting stages, migration, and burrow-

ing into substrates that hold moisture. The fauna of wetlands varies, and among the most ubiquitous macro-fauna are Chironomidae and Dystiscidae (Batzner and Ruhí 2013).

In contrast with wetland habitats, caves and other subterranean habitats are typically among the most environmentally stable freshwater ecosystems (Poulson 1964, Culver and Pipan 2009). Cave streams dry up well after surface streams because they are fed by shallow subsurface aquifers in the epikarst (Williams 2008). Nevertheless, not all aquatic cave habitats are permanent. Many drip pools in caves, perched well above any streams, are subject to periodic drying either because of increased evaporation in cave passages, especially during winter summer months, or because of small changes in the flow path of water moving through epikarst. The epikarst itself, which is not accessible to direct observation or sampling, harbors both terrestrial and aquatic species (Culver and Pipan 2014). This outfall of animals suggests that habitats may switch between aquatic and terrestrial with water movement. Ginet (1960) and Mathieu and Turquin (1992) point out that the drying of pools is a typical part of the environment of some cave-dwelling *Niphargus* amphipods. The subterranean macro-fauna is less varied than that of wetlands, and is dominated by crustaceans, especially amphipods and isopods (Gibert and Culver 2009).

Burrowing to avoid desiccation is well known among wetland invertebrates, especially among species that have no resting stage and are unable to disperse, such as amphipods (Wiggins et al. 1980; DiSalvo and Haynes 2015). However, there are few reports of burrowing behavior of cave amphipods. Ginet (1960) reported burrowing in the subterranean amphipod *Niphargus virei*, an inhabitant of isolated pools in caves in France, and Mathieu and Turquin (1992) summarized his observations. Gounot (1960) suggested that burrowing was actually the result of ingestion of clay for its nutritional value, perhaps some micronutrient given the generally low level of organic carbon in clay. Holsinger and Dickson (1977) observed burrowing behavior in *Cranogonyx antennatus*, a cave pool inhabitant in Virginia caves, in the laboratory, of both populations from permanent and temporary cave waters. The temporary cave waters in this case were epikarst drip pools.

While there is little discussion of wetlands in the cave and karst literature (see Pipan and Culver 2012, Sheehy Skeffington and Gormally 2007, Skeffington and Scott 2008), and even less discussion of caves and karst in the wetlands literature (Reynolds 2016), there is a unique habitat that bridges the two categories – the hypotelminorheic. First described by Meštrović (1962), it is in essence, a superficial subterranean drainage (Pipan et al. 2012) and a miniature wetland at its exit. This definition was later expanded by Culver and Pipan (2014) to include:

- A persistent wet spot that may dry up seasonally
- Underlain by clay or other impermeable layer
- Water exiting via seepage flow, typically from a slight depression
- Presence of blackened leaves
- A subterranean fauna, with species lacking or having reduced eyes and pigment

The hypotelminorheic is a subterranean habitat because it is aphotic except at its exit at a seep or seepage spring, where some light penetrates the leaf litter. We follow Williams' (2017) distinction between seeps and springs. He defines seeps as comprising groundwater that emerges over a more diffuse area [than springs], with a rate of flow that is generally insufficient to form an outflow spring. The exit point, or seep, is an isolated wetland, rich in organic carbon from decaying leaves, and is in turn habitat for amphipods. This, in many ways unremarkable habitat, is made remarkable by its fauna. Of the 56 species in the exclusively subterranean amphipod genus *Stygobromus* found in the eastern United States 12 (21%) are found primarily in hypotelminorheic habitats (Culver et al. 2010). In turn, amphipods and isopods are the dominant macro-organisms of all studied hypotelminorheic habitats (Pipan et al. 2012). Many of these species are both troglomorphic (reduced or absent eyes and pigment, and elaborated appendages) and stygobiotic (limited to subterranean habitats).

With increased monitoring and sampling of seeps in the mid-Atlantic region of the U.S. (e.g., Culver et al. 2012), it became apparent that most seeps have no flow during the summer months (May to October). Whether the subterranean hypotelminorheic habitat also dries out is not directly known, but if the exit to these small aquifers is dry, then the amount of free water in the aquifer is likely reduced. During these drying periods, water is likely retained in the clay layer. The two key properties of clay are both its high porosity (nearly as high as soil) and high specific retention (Heath 1983). Given the water retention of clay, it is plausible that the amphipod and isopod inhabitants of hypotelminorheic habitats burrow into clay as a refuge during dry periods.

In the course of sampling seeps in parklands of Washington, DC, specifically in National Capital Parks-East (Piscataway, Oxon Cove, Anacostia, Fort Dupont, Shepherd Parkway, and others), it was found that the macro-crustacean fauna was dominated by two species of amphipods – *Crangonyx shoemakeri* and a *Stygobromus* species in the *tenuis* group – and the isopod *Caecidotea kenki*, (Keany et al. 2018). The *Stygobromus* species lacks eyes and pigment, and *C. kenki* and *C. shoemakeri* have reduced eyes and pigment. The undescribed species of *Stygobromus* is likely limited to the Anacostia River drainage in Washington, DC, and adjoining parts of Maryland. *C. shoemakeri* is a common inhabitant of wetlands in National Capital Parks-East and elsewhere in its range from southcentral Maryland to southcentral Virginia (Zhang and Holsinger 2003). While not adapted to subterranean life in any obvious way, *C. shoemakeri* has likely adapted to the periodic drying of seasonal wetland habitats where it is found. Occasionally, a third amphipod is found in seeps of the DC metro area, especially those with direct connections to permanent surface streams – *Gammarus minus*. *G. minus* is primarily found in springs, and a distinct morphological variant – *Gammarus minus* var. *tenuipes* – is common in cave streams (but not cave pools, Culver et al. 1995). It is rarely, if ever, a permanent inhabitant of hypotelminorheic habitats. The isopod *Caecidotea kenki* is found in seeps and other small groundwater fed habitats. It is known from the lower Potomac drainage around Washington, DC, as well as two caves in Pennsylvania (Culver et al. 2012). It is not considered further in this study, as our study species were limited to amphipods.

Our goal in this study was to determine whether the ability to burrow into clay substrates is an important factor in determining the presence of *Stygobromus* species and *Crangonyx shoemakeri* in seeps and the general absence of *Gammarus minus*. In order to investigate this, we conducted laboratory experiments monitoring the behavior and success of the three amphipods when water was systematically removed from a clay-filled petri dish.

Methods

Species

Three species were studied – *Crangonyx shoemakeri* (Hubricht and Mackin), *Gammarus minus* (Say), and *Stygobromus tenuis potomacus* (Holsinger). *S. tenuis potomacus* was used rather than the undescribed species from the *tenuis* group because there were not sufficient specimens of the undescribed species available. The two species are very similar morphologically and ecologically. All three species were found in seeps in the parklands of the District of Columbia, although *G. minus* was only found in seepage springs, typically with a permanent outflow, and usually during the Spring when water levels were at their highest.

Specimens used in this study were taken from seeps and springs in C & O Canal National Historic Park under permit CHOH-2016-SCI-0023. These individuals were obtained using standard collecting procedures as described by Holsinger (1972). Early trials were completed using animals from Oxon Run in National Capital Parks-East under permit NACE-2016-SCI-0002. Unused animals were returned to their habitat.

Laboratory methods

The behavioral study was conducted in a Forma-lab Walk-in Environmental Room with a constant temperature of 5 °C. Glass pyrex dishes, with a volume of approximately 470 mL, (10 cm diameter and 4 cm depth) were lined with 30g of dry VWR™ clay, creating a clay layer approximately 2 cm deep. This clay was homogenized using a mortar and pestle and was sifted through a 125µm mesh sieve to ensure consistent grain size. Sixty mL of Deer Park™ spring water were added to each of the glass pyrex dishes, creating a water depth of approximately 2 cm.

The three species of amphipods – *S. tenuis potomacus*, *C. shoemakeri* and *G. minus* – were studied separately. Within one week from the date of specimen collection, five individuals, all adults, of the same species were added to each dish. Ovigerous females were not used. After a 48 hour period of acclimation to the pyrex dishes, the trials began. This acclimation period was chosen based on the 2–10 day amphipod acclimation period recommended by Environment Canada (1993) and Swartz et al. (1985).

Six dishes were designated for each species; three served as the control while the other three were used as experimental dishes that simulated drying conditions. Each trial lasted five weeks.

A supplemental experiment was completed to determine the amount of water loss (retention) of the clay over the course of eight weeks. The dry weight of the clay was compared to the weight of the clay after one to eight weeks of drying. Measurements began at the end of week 1, after all surface water was removed.

During the course of the drying experiment, behavioral observations were made for 60 second intervals three times a week. The following behaviors were recorded if one or more individuals was observed with this behavior during the 60 second interval:

- 1 Walking (at least one body length)
- 2 Swimming (at least one body length)
- 3 Burrowing (direct observation or buried in clay)
- 4 Resting (resting for more than 10 seconds)
- 5 Interaction (with other amphipods)
- 6 Other (amplexus, etc.)

The relative frequency of these behaviors is not a direct measure of the time spent at each behavior because a behavior was given the same weight whether one or five amphipods was displaying the behavior. This indirect measure was necessary because it was impossible to track each individual separately. All observations were made under red light.

Data analysis

Both survival and behavioral data were analyzed using log-likelihood tests (G-tests), comparing observed and expected values in each category (e.g., walking in control dishes). G, which is distributed as χ^2 was calculated as follows:

$$G = 2 \sum_i O_i \ln \left(O_i / E_i \right)$$

where O_i is the observed number of cases in category i and E_i is the expected number of cases in category i .

Results

Gravimetric measurement of moisture levels of the clay at the start of each trial averaged at 65.5% and reduced to an average of 54.2% after the first week of drying.

Table 1. Results of experiments on effect of drying. All runs began with 15 individuals.

Species	Start of Trial	Control-surviving at 4 weeks	Experiment-surviving at 4 weeks	Difference	Log-likelihood	p
<i>Gammarus minus</i>	7/20/2016	15	8	-7.00	5.15	<0.05
	7/20/2016	15	7	-8.00	4.44	<0.05
	8/9/2016	15	10	-5.00	3.45	<0.10
	1/8/2017	2	2	0.00	0.00	ns
	1/8/2017	1	2	1	0.27	ns
	Total	48	29	-19	7.61	<0.010
<i>Crangonyx shoemaker</i>	7/20/2016	13	12	-1	0.53	ns
	8/9/2016	15	15	0	0.00	ns
	1/8/2017	15	15	0	0.00	ns
	1/8/2017	15	15	0	0.00	ns
	1/8/2017	12	15	3	1.95	ns
	Total	70	72	2	0.25	ns
<i>Stygobromus tenuis potomacus</i>	7/20/2016	14	15	1	0.62	ns
	1/8/2017	15	15	0	0.00	ns
	1/8/2017	15	15	0	0.00	ns
	Total	44	45	1	0.61	ns

Moisture levels then reduced to 34.4% at week two, and remained relatively consistent from week two to week nine, at around 30% (Fig. 1).

Overall, there was a 25% increase in mortality for *G. minus* when subjected to drying (Table 1), which was a statistically significant difference between experiment and control ($G=7.61$, $p<0.01$). The difference was especially striking in the first three trials where no control animals died, and 44% of the amphipods died in the experimental treatment. In the last two trials, only a few amphipods survived (23%) in either experiment or control treatments. We cannot account for this difference between the first three and the last two experiments, but the high mortality suggests some water quality problem. However, no increased mortality was recorded for the other species. Both *C. shoemakeri* and *S. tenuis potomacus* showed the identical pattern of little or no mortality in either experiment or control (Table 1). For *C. shoemakeri* only 4% of experimental animals died, slightly fewer than the number of control animals that died. For *S. tenuis potomacus*, there was no mortality in the experimental treatments, and only one amphipod died in the control (Table 1). Thus, only *G. minus* had significant mortality associated with the drying experiment, and there were no differences in the survival of *C. shoemaker* and *S. tenuis potomacus*.

The three species showed differences in behavior, both between experiment and control, but also between species (Fig. 2, Table 2). In the controls, all three species showed frequent interaction (between 30% and 40% of the observational periods had one or more interactions) with other individuals, typically avoidance behavior (see Culver 1970). Locomotory behavior was observed about half the time, typically swimming in the case of *G. minus* and walking in the case of *C. shoemakeri*. Somewhat surprisingly, *S. tenuis potomacus* was equally likely to swim or walk, even

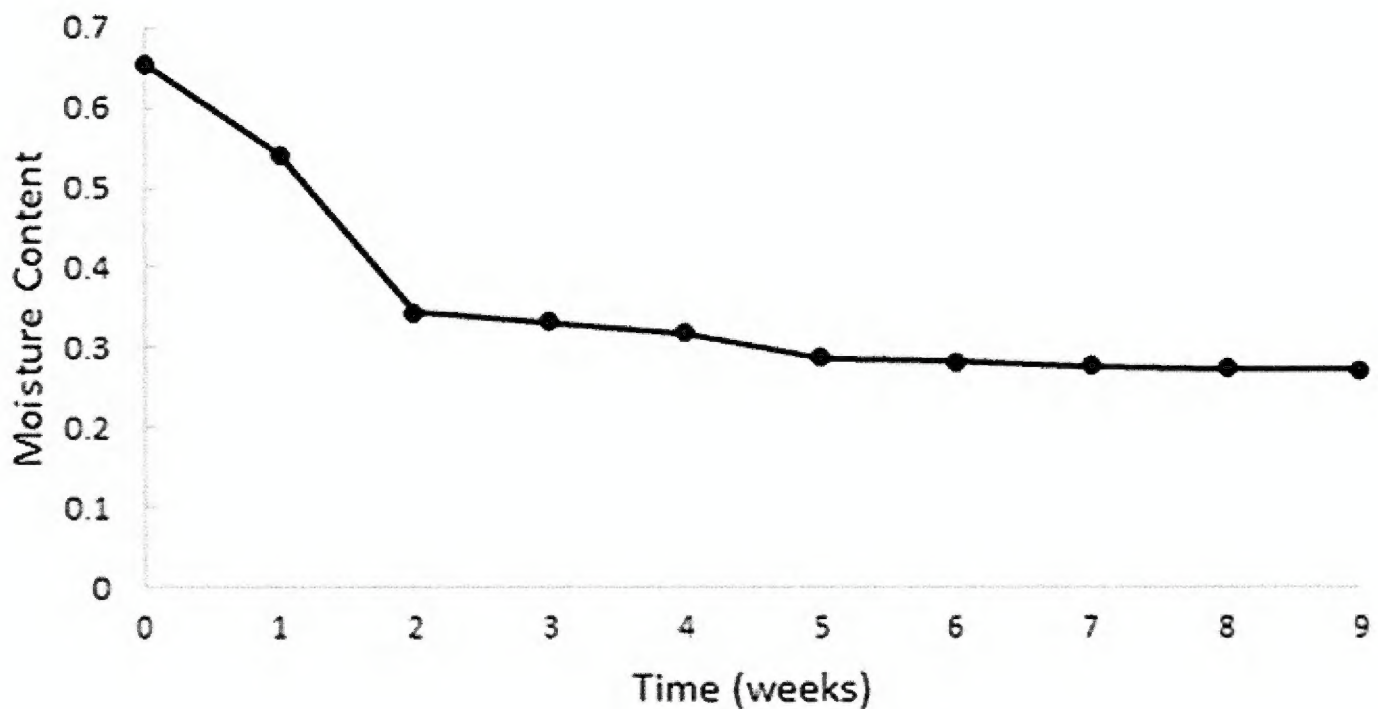


Figure 1. Average gravimetric soil moisture over the nine week trial.

though species in the genus are weak swimmer and usually walk (Culver 1970). Resting was uncommon in all three species in control containers, occurring less than 10% of the time (Fig. 2). *G. minus* were in amplexus 12 % of the time. This behavior was particularly frequent in the last two control trials. Overall, in the control containers, the three species had differences in behavior (Table 3) due to species specific behavioral patterns in locomotion (swimming vs. walking) and amplexus (only with *G. minus*).

When in the experimental containers, each species showed statistically significant differences in behavior relative to the control containers (Table 3). *G. minus* showed decreases in all categories except inactivity, which was observed 85% of the time (Fig. 2, Table 2). Typically, the *G. minus* animals were stretched out on the substrate and burrowing was only observed once. Amplexing was a particularly frequent behavior in the last two trials, perhaps due to seasonality of mating behavior.

For *C. shoemakeri*, resting was also the most common activity, occurring 77% of the time (Fig. 2). In comparison with resting *G. minus*, resting *C. shoemakeri* curled into 'C' shapes keeping their appendages tight to their bodies. Burrowing, and residence in burrows was the second most common activity, occurring 10.4% of the time. Not surprisingly, swimming almost completely disappeared (less than 1%) and walking was observed only 9.4% of the time, given that there was no open water (Fig. 2).

Overall, *S. tenuis potomacus* showed less behavioral change than the other two species, but the differences were statistically significant compared to its control group (Table 2). Resting was the most common behavior in the experimental conditions, occurring 42.1% of the time, but was still less frequent when compared to resting in the other two species. Walking and interaction remained common, although less so than in controls,

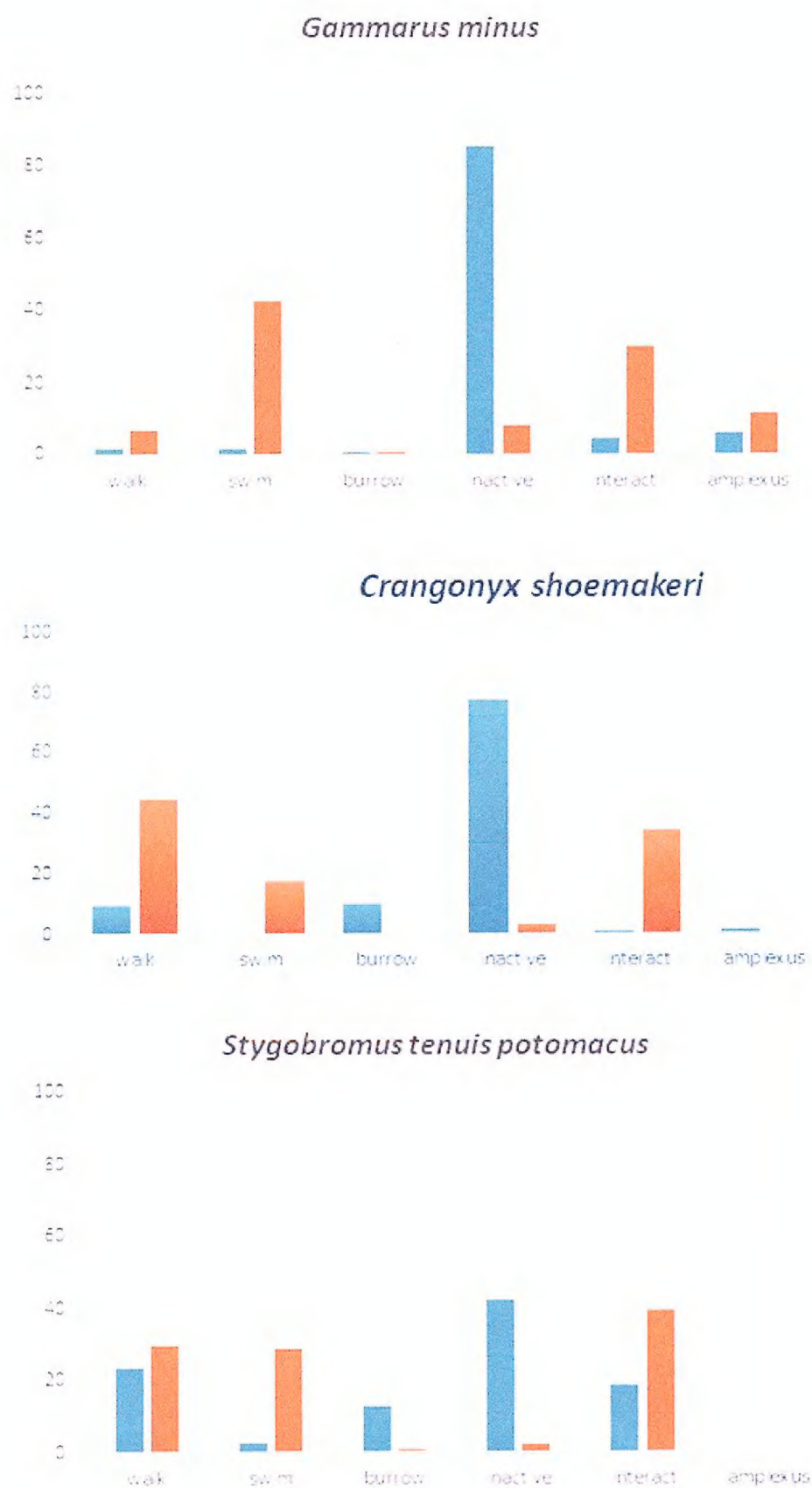


Figure 2. Relative frequency of different behaviors of the three species in control (red bars) and experimental (blue bars).

and swimming declined to less than 3% (Fig. 2, Table 2). Burrowing (and residence in burrows) was relatively frequent in *S. tenuis potomacus*, accounting for 12.9% of observations in the experimental group. The burrows of *S. tenuis potomacus* were deeper than those of *C. shoemakeri*, but they both had a similar C-shape when resting. *S. tenuis potomacus* leave circular depressions visible from the top of the soil where they have entered the clay. They exhibit the greatest amount of burrowing and travel the deepest into the clay; one *S. tenuis potomacus* hole was visible from the bottom of the dish.

Table 2. Number of observations of six different behaviors of *Crangonyx shoemakeri*, *Gammarus minus*, and *Stygobromus tenuis potomacus* in experimental and control containers. If at least one individual (and up to five) shows the behavior, this is counted as an observation. See methods for the observational protocols.

Category	<i>Gammarus</i>		<i>Crangonyx</i>		<i>Stygobromus</i>	
	Exp	Control	Exp	Control	Exp	Control
1- walk	3	20	19	138	40	55
2-swim	3	125	1	54	5	53
3-burrow	1	2	21	0	22	1
4-resting	161	24	156	10	72	4
5-interact	8	88	2	106	32	73
6-amplexus	11	34	3	0	0	0
Total	189	293	202	308	171	186

Table 3. Log-likelihood tests for differences between behaviors in experimental and control containers for *Crangonyx shoemakeri*, *Gammarus minus*, and *Stygobromus tenuis potomacus*, and among control behavior and among behavior in experimental containers. n_1 , n_2 , and n_3 are the total number of observations of a behavior, e.g., walking. The total number of separate observations was 180 for *G. minus* and *C. shoemakeri*; 108 for *S. tenuis potomacus*. For some observation periods more than one behavior was observed. See Figure 1.

Comparison	n_1	n_2	n_3	df	log - likelihood	p
<i>G. minus</i> -experiment vs. control ²	188	291		4	147.6	<0.01
<i>C. shoemakeri</i> -experiment vs. control ¹	199	308		4	198.9	<0.01
<i>S. t. potomacus</i> -experiment vs. control ¹	171	186		4	70.4	<0.01
All control	308	291	186	10	112.1	<0.01
All experimental	199	188	171	10	70	<0.01

¹Amplexus is excluded due to small sample size.

²Burrowing is excluded due to small sample size.

Discussion

Adaptation to the absence of open water

Crangonyx shoemakeri and *Stygobromus tenuis potomacus* have evolved mechanisms allowing for their success in habitats that periodically lack open water. Both of these species exhibited similar adaptations by burrowing into the substrate during experimental drying periods. Those individuals that failed to burrow, remaining exposed on the surface, curled in ‘C’ shapes and often times lay in shallow depressions. The length of time that burrowing in clay can provide a refuge from desiccation is unknown, beyond the four week duration of the experiment. We do know that moisture is retained in clay for longer periods (see Fig. 1).

Animals living in surface habitats that periodically dry, including many wetlands, have a large array of strategies to cope with desiccation (Wiggins et al. 1980). *Crangonyx* is a frequent inhabitant of wetlands (Boix and Baxter 2016), and Wiggins et al. (1980) report that *C. gracilis* uses crayfish burrows as refuges from desiccation. Wiggins et al. (1980) report that *C. rivularis* burrows into sediments and bottom vegeta-

tion during the absence of open water in summer and autumn. DiSalvo and Haynes (2015) describe an interesting strategy employed by *C. pseudogracilis* in temporary pools. During wet weather they actively enter existing small tubes in the soil, and are able to persist throughout the year in damp soil. Unfortunately, no phylogeny exists for *Crangonyx* and so we cannot look directly at evolutionary relationships of this behavior.

In addition to its widespread occurrence in wetlands, *Crangonyx* is also a frequent inhabitant of caves and some of these species occur in epikarst drip pools, a subterranean habitat that occasionally dries out (Pipan and Culver 2013). Holsinger and Dickson (1976) observed burrowing behavior in an epikarst drip pool population of *Crangonyx antennatus* in Molly Waggles Cave in Virginia.

The only other observations of burrowing behavior in subterranean habitats comes from the European amphipod genus *Niphargus*. Ginet (1960) observed *Niphargus* curling into a 'C' shape in a shallow burrow where individuals were at least partially visible from the surface (see also Mathieu and Turquin 1996). Interestingly, they reported that an air bubble was typically present on the dorsal side, presumably aiding in oxygen exchange. It remains unknown if either *Stygobromus tenuis potomacus* or *Crangonyx shoemaker* form such air bubbles, but none were directly observed. Gounot (1960) asserted that the clay supplies important nutrients from the presence of inert organic substances as well as microorganisms. She based this on experiments where survival was enhanced when *Niphargus virei* was provided a clay substrate compared to the control where no substrate was provided. Gounot showed that while the nutrients associated with clay were needed for immature *Niphargus*, they are not sufficient as the only source of nutrients for adults. To our knowledge, these preliminary experiments have never been followed up.

Conservation and protection

Clay substrates provide important resources for amphipod species from nutrition to surviving environmental stressors. Our findings indicate the importance of a clay layer for *Crangonyx shoemakeri* and *Stygobromus tenuis potomacus* amphipods inhabiting hypotelminorheic habitats in the Washington, DC region. To protect these species and the endemic and endangered species, *Stygobromus hayi*, it is essential to maintain healthy soil. Although these cryptic species may not be found during drying periods, it is apparent that the animals are still present. Due to the difficulty in accessing the microenvironments these animals create in the soil, biological surveys may report false negatives. The application of eDNA sampling holds promise for detecting these populations (Niemiller 2017).

By 2090, it is expected that the proportion of the global land surface in extreme drought will increase tenfold from current levels (Kundzewicz et al. 2008). Due to the extension of droughts and decreasing water levels, wetlands are at risk. While some of these wetlands are ephemeral and therefore not always wet, they constitute a large portion of the Earth's freshwaters and support freshwater biodiversity (Williams 2006). As a result of wetland global significance to healthy ecological systems, climate-driven changes to wetland hydrology have been identified as a key global wetland conservation issue (Klein

et al. 2005; Waterkeyn et al. 2008). Records of river and wetland hydroperiods already indicate pronged and more frequent dry periods (Strachan et al. 2014). These measurements are two components of a hydroregime. The characteristics of a particular hydroregime, particularly the hydroperiod, have a direct influence on aquatic community structure and its biodiversity (Brooks 2000; Williams 2006; Vanschoenwinkel et al. 2009).

In Australia, Sim et al. (2013) examined long-term data sets of temporal trends in the persistence and the stability of freshwater ecosystems and documented changes in wetland hydroperiods over the past 50 years. At one site, the hydroperiod was significantly correlated with invertebrate community richness. Ultimately, however, the study could not capture the true effects of long term drying. The magnitude of drying on these communities is yet to be fully recorded. As successive hydroperiods decrease without sufficient time and recharge, the flexibility of invertebrate survival strategies will be tested. Small wetlands that facilitate movement of species on a landscape scale may completely dry, leaving exposed the many species that rely on migrating to nearby permanent water after ephemeral habitats dry (Sim et al. 2013). This loss of water is cited as one of the most deleterious of environmental changes, changing the patch dynamics of species. As species geographic range is reduced, metapopulations will be altered (Robson et al. 2011). If surface water disappears completely, this would signify the end of the corresponding habitat niche, and only those inhabitants capable of surviving without even intermittent surface water could persist in the long term.

In order to further assess the impact these anthropogenic changes to the environment will have on amphipod species, it is necessary to learn more about their physiological sensitivity. To better understand amphipod adaptations and estimate the impact of warming on their fitness, further studies on the burrowing behavior of *Crangonyx shoemakeri* and *Stygobromus tenuis potomacus* should be investigated.

Acknowledgements

This research was supported by Chesapeake Watershed Cooperative Ecosystem Studies Unit Cooperative Agreement P16AC01389 between Department of the Interior, National Park Service, National Capital Parks – East and American University, “A Survey of the Fauna of Seepage Springs in National Capital Parks-East (NACE)”. All specimens were collected under CHOH-2016-SCI-0023 and NACE-2016-SCI-0002 permits. A reviewer offered many constructive comments.

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